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Site-directed mutagenesis identifies a tyrosine radical involved in the photosynthetic oxygen-evolving system

(protein engineering/water oxidation/electron transfer/EPR spectroscopy/cyanobacteria)

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ABSTRACT **Photosynthetic oxygen evolution takes place in the thylakoid protein complex known as photosystem II. The reaction center core of this photosystem, where photochemistry occurs, is a heterodimer of homologous polypeptides called D1 and D2. Besides chlorophyll and quinone, photosystem II contains other organic cofactors, including two known as Z and D. Z transfers electrons from the site of water oxidation to the oxidized reaction center primary donor, $P_{680}^{+\cdot}$, while $D^{+\cdot}$ gives rise to the dark-stable EPR spectrum known as signal II. $D^{+\cdot}$ has recently been shown to be a tyrosine radical. Z is probably a second tyrosine located in a similar environment. Indirect evidence indicates that Z and D are associated with the D1 and D2 polypeptides, respectively. To identify the specific tyrosine residue corresponding to D, we have changed Tyr-160 of the D2 polypeptide to phenylalanine by site-directed mutagenesis of a *psbD* gene in the cyanobacterium *Synechocystis* 6803. The resulting mutant grows photosynthetically, but it lacks the EPR signal of $D^{+\cdot}$. We conclude that D is Tyr-160 of the D2 polypeptide. We suggest that the C_2 symmetry in photosystem II extends beyond P_{680} to its immediate electron donor and conclude that Z is Tyr-161 of the D1 polypeptide.**

In plants, algae, and cyanobacteria, light initiates electron transfer reactions that generate the chemical free energy and reducing equivalents required for biosynthesis and CO_2 fixation. These reactions take place in the thylakoid membrane and involve several multisubunit protein complexes including photosystems I and II, the cytochrome b_6f complex, and ATP synthase (for review, see refs. 1 and 2). Photosystem II (PSII) couples light-induced charge separation with the reduction of plastoquinone and the oxidation of water (for review, see refs. 3 and 4). Molecular oxygen is released as a waste product of the water-splitting reactions. Of the seven polypeptides that comprise the minimal unit of PSII, attention has focused on two of these, D1 and D2, as binding key elements of the photochemical apparatus (5–7). These include P_{680}, a specialized monomer or dimer of chlorophyll (Chl) that serves as the light-induced electron donor, and the pheophytin and quinone electron acceptors. The D1 and D2 polypeptides have molecular masses in the 30-kDa range and exhibit sequence similarity with each other and with the L and M subunits of reaction centers from purple nonsulfur bacteria. These similarities and the C_2 symmetry present in the crystallographic structure of the reaction centers of *Rhodopseudomonas viridis* (8) and *Rhodobacter sphaeroides* (9) have led to the suggestion that a similar symmetry is present in the D1/D2 core (8, 10–13).

Although the precise mechanism of oxygen evolution by PSII is unknown, a cluster of four Mn atoms accumulates the oxidizing equivalents necessary for water splitting by donating electrons to the oxidized primary donor $P_{680}^{+\cdot}$. These electron transfers proceed through an intermediate charge carrier usually designated as Z. The $Z^{+\cdot}$ species is EPR detectable and has a spectrum identical to the stable PSII free radical, $D^{+\cdot}$, that gives rise to the well-known signal II line shape (3). While the function of D remains obscure, recent EPR data have shown that $D^{+\cdot}$ is a tyrosine radical (14). Owing to the spectral similarity between $Z^{+\cdot}$ and $D^{+\cdot}$, it seems likely that $Z^{+\cdot}$ is also a tyrosine radical, located in an environment similar to that of $D^{+\cdot}$ (14). The identities of the polypeptides that contain Z and D are uncertain, but indirect kinetic evidence indicates that Z is associated with the same polypeptides that bind P_{680} (15) and recent iodination data imply that Z may be located on D1 and that D may be located on D2 (16, 17).

If Z and D are both tyrosines in similar environments, with Z on D1 and D on D2, the symmetry of the D1/D2 model for PSII implies that there may be a tyrosine conserved between these two polypeptides. In fact, there are two such tyrosines. In the folding patterns for D1 and D2 proposed by Trebst (13), one tyrosine is located on the stromal side of the membrane in the region of Q_AFeQ_B, while the second, Tyr-160, is located in a region of helix C highly conserved between D1 and D2, near His-197 of helix D, a putative P_{680} ligand.

We propose that D is Tyr-160 of D2 and that Z is the corresponding Tyr-161 of D1. To test the first part of this hypothesis, we have changed Tyr-160 of the D2 polypeptide to phenylalanine by site-directed mutagenesis in the unicellular cyanobacterium *Synechocystis* 6803. This organism has two copies of the gene corresponding to D2, *psbD-1* and *psbD-2* (18). In this study, we have deleted the 3′ 70% of *psbD-2* from the *Synechocystis* 6803 genome and subsequently mutagenized *psbD-1* by converting Tyr-160 to Phe-160. The resulting mutant grows photosynthetically, but its EPR spectrum is devoid of the $D^{+\cdot}$ line shape. We conclude that the D2 residue, Tyr-160, is D. By analogy, we conclude that Tyr-161 of D1 is Z.

MATERIALS AND METHODS

DNA Manipulations. All manipulations of DNA, including restriction endonuclease analyses, Southern blotting, and DNA hybridizations with [^{32}P]DNA were performed according to standard protocols (19), except as noted.

Growth of Synechocystis. A glucose-tolerant strain of *Synechocystis* 6803 (20) was grown as described (20) in constant light at 33°C in BG-11 medium (21) supplemented with 5 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), pH 8.0. Solid medium was BG-11 supplemented with 1.5% (wt/vol) agar/10 mM TES, pH 8.0/0.3% (wt/vol) sodium thiosulfate/5 mM glucose. When appropriate, media

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Abbreviations: Chl, chlorophyll; Cm, chloramphenicol; Km, kanamycin; PSII, photosystem II.

were supplemented with 2.5 μg of chloramphenicol (Cm) per ml or 2.5 μg of Cm plus 5 μg of kanamycin (Km) per ml. Total genomic DNA was extracted essentially as described (20).

Deletion of *psbD-2*. There are two copies of *psbD* in *Synechocystis* 6803 (18). The sequences of both are known, and both coding regions are 1056 base pairs (bp) long (ref. 18; see Fig. 1). The 3′ terminus of *psbD-1* overlaps *psbC* (encoding CP43, another polypeptide present in PSII) by ≈50 bp (18). Consequently, we chose to delete *psbD-2* from the *Synechocystis* genome and to mutagenize *psbD-1*.

psbD-1 was originally cloned into pUC19 (22) as an 8.8 kilobase (kb) *Kpn* I/*Eco*RI genomic fragment by J. G. K. Williams in this laboratory in 1983. With *psbD-1* as a probe, *psbD-2* was isolated from a λ EMBL-3 library prepared from a *Sau*3A partial digest of total *Synechocystis* DNA. A 1.4-kb *Hin*cII fragment, bearing the 3′ 90% of *psbD-2*, was subcloned into pUC19. A 745-bp *Bst*EII/*Sma* I fragment (positions 295–1040 in *psbD-2*; see Fig. 1*A*) was replaced with a 1.9-kb fragment from pKT210 (23) (isolated from pRL171; the kind gift of J. Elhai and P. Wolk, Michigan State University, East Lansing, MI) bearing a bacterial gene encoding resistance to Cm. *Synechocystis* 6803 was transformed with the altered plasmid as described (20), and transformants were selected for ability to grow on Cm. Southern blot analyses of genomic DNA from selected transformants confirmed that the wild-type 1.4-kb *Hin*cII fragment of *psbD-2* had increased to 2.6 kb.

Mutagenesis. The original genomic DNA fragment bearing *psbD-1* contains ≈4 kb of sequence upstream of *psbD-1*. To generate several unique restriction sites in and near *psbD-1*, the 2.5 kb farthest upstream from *psbD-1* was removed. A 1.25-kb fragment of pUC4K (24), bearing a gene encoding resistance to Km, was inserted into an *Eco*RV site 800 bp upstream of *psbD-1* (generating pRD1219Km^r; see Fig. 1*B*).

Tyr-160 of *psbD-1* in *Synechocystis* 6803 has a TAC codon centered at position 479. This codon provides part of a recognition site for the restriction endonuclease *Rsa* I (5′ GTAC 3′). The mutation TAC → TTC changes Tyr-160 to Phe-160 and eliminates the *Rsa* I site. At positions 295 and 738 of *psbD-1* are two nonequivalent *Bst*EII sites (see Fig. 1*B*). The codon for Tyr-160 thus lies on a 443-bp *Bst*EII fragment; the mutation changing Tyr-160 to Phe-160 eliminates an *Rsa* I site within this fragment.

A synthetic 15-base oligonucleotide (5′ TTGATGTTCCCCTTG 3′), centered at position 479, and containing the single A → T base change, was synthesized by the solid-state phosphorimidate method with an Applied Biosystems model 380A DNA synthesizer. Packaged single-stranded template DNA was generated by superinfecting cells containing pUC118, bearing the 1.7-kb *Xba* I/*Eco*RI fragment of pRD1219Km^r (see Fig. 1*B*), with the helper phage M13K07 (30). Double-stranded DNA bearing the A → T conversion was generated with the Amersham oligonucleotide-directed mutagenesis system that preferentially degrades the wild-type DNA strand. After transformation into *Escherichia coli* (strain TG-1 from Amersham), restriction analysis of plasmid DNA isolated from eight transformants showed that two transformants carried pure mutant plasmid, lacking the *Rsa* I site at position 479. Subsequent DNA sequence analysis by the dideoxynucleotide chain-termination method (25) showed no mutations within the 443-bp *Bst*EII fragment of *psbD-1* other than the desired A → T conversion at position 479.

To introduce the mutation into *Synechocystis* 6803, the wild-type *Bst*EII fragment of *psbD-1* within pRD1219Km^r (see Fig. 1*B*) was replaced with the *Bst*EII fragment bearing the A → T conversion. The resulting mutation-bearing plasmid was then transformed into the *Synechocystis* strain having the 3′ 70% of *psbD-2* replaced by the gene encoding resistance to Cm. Transformants were selected for resistance to both Km and Cm on solid medium containing glucose. The resulting Km^r/Cm^r transformants should contain either the mutant *psbD-1* or the wild-type *psbD-1*, depending on whether the A → T conversion at position 479 was cotransferred with the gene encoding resistance to Km. Transformants carrying the altered gene can be differentiated from those carrying the wild-type gene on the basis of the *Rsa* I site present in the wild-type *psbD-1* but absent from the mutant.

EPR Spectra and O_2 Evolution. Cells were pelleted and resuspended in 7.5% (wt/vol) polyethylene glycol 3400 (Aldrich)/20 mM Hepes/1 mM EDTA, pH 7.5. EPR spectra and spin quantitations were obtained as described (14, 15), with a Bruker ER200D spectrometer operated with a Varian TM cavity. Oxygen evolution was measured at 25°C in a glass cuvette fitted with a Clark-type O_2 electrode (Yellow Springs Instrument) with saturating white light provided by two high-intensity microscope illuminators fitted with heat filters. Cells (15 μg of Chl) were diluted into 3.5 ml of 20 mM Hepes, pH 7.5/50 mM NaCl/5 $MgCl_2$/1 mM 2,6-dichlorobenzoquinone.

RESULTS

Construction of Phe-160 Mutant. *Synechocystis* 6803 cells lacking a functional *psbD-2* (Fig. 1*A*) were transformed with pRD1219Km^r bearing the desired A → T conversion at position 479 of *psbD-1*. Total genomic DNA from eight Km^r/Cm^r transformants was digested with *Hin*cII, size-separated by electrophoresis, and transferred to nitrocellulose. The resulting Southern blot was hybridized with a ^{32}P-labeled plasmid bearing the 1.4-kb *Hin*cII fragment of *psbD-2*. The results (shown for two transformants in Fig. 2*A*) showed that all transformants contained a 1.2-kb insertion within the 3.4-kb *Hin*cII fragment of *psbD-1* and that none contained the wild-type 1.4-kb *Hin*cII fragment of *psbD-2*.

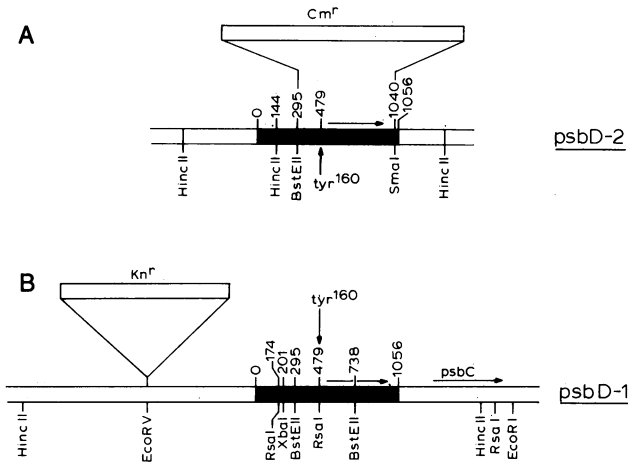

FIG. 1. The 1056-bp *psbD* genes from *Synechocystis* 6803 (18), plus flanking DNA. (*A*) *psbD-2*, showing the 745-bp *Bst*EII/*Sma* I fragment replaced by a 1.9-kb fragment of pKT210 (23) bearing a gene encoding resistance to Cm. (*B*) *psbD-1*, showing the position of the codon encoding Tyr-160, the two nonequivalent *Bst*EII sites that flank this codon, and the three *Rsa* I sites, one at position 174 of *psbD-1*, one at the Tyr-160 codon, and one in *psbC*, used for differentiating between mutant and wild-type *psbD-1* in Km^r/Cm^r transformants (see Fig. 2 and text). The plasmid pRD1219Km^r contains 3.6 kb of *Synechocystis* DNA extending from the *Hin*cII site 1.7 kb upstream of *psbD-1* to the *Eco*RI site within *psbC*. This plasmid also carries a 1.25-kb fragment from pUC4K (24), bearing a gene encoding resistance to Km (Km^r), inserted into the *Eco*RV site 800 bp upstream of *psbD-1*, as indicated.

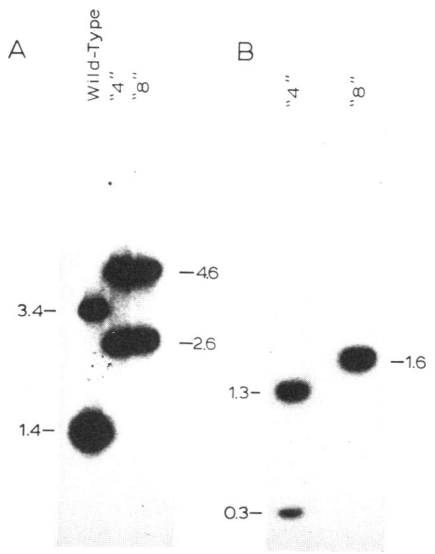

FIG. 2. Southern blot analysis of total genomic DNA from wild-type *Synechocystis* 6803 and two Km^r/Cm^r transformants. Fragment sizes expressed in kb are indicated. (*A*) DNA digested with *Hin*cII and hybridized with ^{32}P-labeled plasmid bearing the 1.4-kb *Hin*cII fragment of *psbD-2*. In the DNA from wild-type cells (left lane), both the 1.4-kb fragment of *psbD-2* and the 3.4-kb fragment bearing *psbD-1* are visible. Both fragments in both transformants are larger, indicating insertion of the 1.2-kb Km^r gene upstream of *psbD-1*, and replacement of the 3' 70% of *psbD-2* by the 1.9-kb Cm^r gene. (*B*) DNA from the two transformants in *A* was digested with *Rsa* I and hybridized with the ^{32}P-labeled 443-bp *Bst*EII fragment of *psbD-1* (see Fig. 1*B*). Transformant 4 shows the two fragments characteristic of native *psbD-1*, having an *Rsa* I site in the Tyr-160 codon. Transformant 8 shows only one fragment, indicating loss of this *Rsa* I site and therefore the presence of the mutant *psbD-1*, having the codon for Tyr-160 replaced by that for phenylalanine.

To differentiate between Km^r/Cm^r transformants carrying mutant and wild-type *psbD-1*, total genomic DNA was digested with *Rsa* I, size-separated by electrophoresis, and transferred to nitrocellulose. The resulting Southern blot was hybridized with the ^{32}P-labeled 443-bp *Bst*EII fragment of *psbD-1*. In cells containing wild-type *psbD-1*, this *Bst*EII fragment should hybridize to two *Rsa* I fragments, one of 300 bp, internal to *psbD-1*, and a second of 1.3 kb, that includes part of *psbC* (see Fig. 1*B*). In cells containing the mutant *psbD-1*, the *Rsa* I site at codon 160 is eliminated. Hence, only one band of 1.6 kb should be observed. Fig. 2*B* shows that transformant no. 4 in Fig. 2*A* contains the wild-type *psbD-1*, while transformant no. 8 contains the *psbD-1* having the desired mutation. Of eight cultures examined, two carried *psbD-1* with the desired mutation, while six carried the wild-type gene.

Photosynthetic Growth and O_2 Evolution. Km^r/Cm^r transformants bearing the mutant *psbD-1* (e.g., transformant no. 8 in Fig. 2) grow photosynthetically but at a slower rate than wild-type cells or Km^r/Cm^r transformants bearing the wild-type *psbD-1*. The mutant cells have a generation time of ≈30 hr, compared to ≈14 hr for cells with wild-type *psbD-1*. Interestingly, we find that the Tyr-160 to Phe-160 mutation has no effect on the rate of O_2 evolution, which is ≈200 μmol of O_2 (mg of Chl)$^{-1}$·hr^{-1} for all cell types. As expected, the mutant grows photoheterotrophically (in the presence of glucose) at normal rates.

EPR Spectra. EPR spectra of Km^r/Cm^r *Synechocystis* cells having wild-type or mutant (Tyr-160 → Phe-160) *psbD-1* are shown in Fig. 3. Cells bearing wild-type *psbD-1* (Fig. 3*A*) show a typical D^+ spectrum with a line width of 19G and a g value of 2.0042. In contrast, in cells bearing the Phe-160

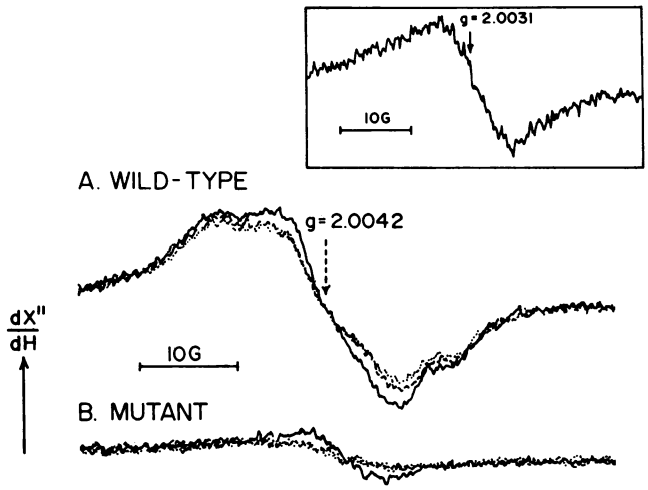

FIG. 3. EPR spectra of photoautotrophically grown Km^r/Cm^r *Synechocystis* cells recorded at room temperature at various times after illumination. (*A*) Cells having wild-type *psbD-1*. (*B*) Cells having mutant *psbD-1* with Tyr-160 → Phe-160. Spectra shown as solid lines were recorded immediately after illumination, dashed spectra were recorded 14 min after illumination, and dotted spectra were recorded 28 min after illumination. Conditions were as follows: frequency, 9.40 GHz; power, 20 mW; gain, 2.0×10^6; field modulation, 3G; amplifier time constant, 500 msec; sweep time, 200 sec. The Chl concentration was 2.6 mg/ml in *A* and 1.7 mg/ml in *B*. (*Inset*) Average of four spectra of the Tyr-160 → Phe-160 mutant, each recorded immediately after illumination (gain, 3.2×10^6), displayed on an expanded scale to show the spectral line shape.

mutation (Fig. 3*B*), this signal is replaced by a signal of lower amplitude with a line width of 10G and a g value of 2.0031 (see Fig. 3 *Inset*). The differences in line shape, line width, and g value clearly distinguish the signal observed in the mutant cells from the spectrum of D^+. Moreover, the signal observed in the mutant corresponds to only 20% of the spins giving rise to the wild-type D^+ signal. Finally, the signal observed in the mutant largely decays within 28 min (dotted spectrum in Fig. 3*B*), while the spectrum of D^+ decays very little during this time period (Fig. 3*A*). Taken together, the data of Fig. 3 demonstrate that the signal of D^+ is absent from the mutant; the signal observed in the mutant comes from a radical other than D^+.

DISCUSSION

To test our hypothesis that component D of PSII is Tyr-160 of the D2 polypeptide, we have changed this tyrosine to phenylalanine by site-directed mutagenesis of a *psbD* gene in the unicellular cyanobacterium *Synechocystis* 6803. The resulting mutant grows photosynthetically and evolves O_2, demonstrating that functional PSII complexes assemble in spite of the mutation. This is in contrast to previous reports of site-directed mutations at His-197 and His-214 of the D2 polypeptide in *Synechocystis* (26, 27).

We have shown that the EPR signal of D^+ is eliminated or substantially diminished in the mutant cells (Fig. 3). This constitutes a strong argument for equating component D with Tyr-160 of the D2 polypeptide. Although we cannot exclude the possibility that component D is some other residue or cofactor perturbed by the mutation, we consider this possibility to be unlikely in view of recent data showing D^+ to be a tyrosine radical (14).

The ability of the mutant cells to grow photosynthetically demonstrates that component D is not essential for PSII function, at least when cells are grown under continuous illumination. Under different growth conditions, component D may serve a crucial role. For example, Styring and

Rutherford (28) have recently proposed that D^{+} may serve to stabilize the Mn cluster during prolonged periods of darkness. Our finding that the steady-state rate of O_2 evolution is the same in mutant and wild-type cells is not surprising in view of the slow kinetic behavior of D^{+} (29). However, the slower photosynthetic growth rate of the mutant indicates that D^{+} may play an important role in the assembly, stability, or long-term function of PSII.

Identification of component D as Tyr-160 of the D2 polypeptide provides further opportunities for probing structure/function relationships in PSII. For example, the EPR spectrum of D^{+} implies that it is a neutral unprotonated radical (14). Perhaps a basic amino acid forms a hydrogen bond with Tyr-160 and accepts its hydroxyl proton upon formation of D^{+}. Comparison of the proposed folding patterns of D1 and D2 (13) with the crystallographic structure of the reaction center from *Rb. sphaeroides* (9) leads us to suggest His-189 of D2 as a possible candidate for such a hydrogen-bond acceptor. Changing this residue to another of stronger or weaker basicity, or changing Tyr-160 to another potentially oxidizable residue, such as cysteine, histidine, methionine, or tryptophan, may shed light on factors influencing the redox potential and function of D.

Although the role of D^{+} in water oxidation is uncertain, the function of Z in electron transfer is well established. The g values, partially resolved hyperfine structures, and orientation dependences of the EPR spectra of Z^{+} and D^{+} are remarkably similar. These observations led to the identification of Z^{+} as a tyrosine radical (14). Here, we extend this hypothesis by assigning Z to Tyr-161 of the D1 polypeptide. Not only is this particular residue conserved between D1 and D2 but so are the four preceding and four following amino acids in the sequences (with the single exception of the conservative replacement of a leucine in D2 by an isoleucine in D1). This high degree of sequence similarity in the Tyr-160/161 region of D1 and D2 provides the similarity in local environment that is demanded by the spectral similarities of Z^{+} and D^{+}. Identification of D as Tyr-160 of D2 and Z as Tyr-161 of D1 indicates that the C_2 symmetry apparent in the bacterial reaction center, and likely to exist in PSII, extends beyond P_{680} to its immediate electron donor.

Note Added in Proof. Results similar to ours have recently been obtained with a *Synechocystis* 6803 mutant having an independently constructed Tyr-160 → Phe-160 mutation in the D2 polypeptide (W. F. J. Vermaas, Ö. Hansson, and A. W. Rutherford, personal communication).

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